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Award Number: DAMD17-98-1-8271

TITLE: The Tumor Suppressor Protein TEPl/PTEN/MMAC1 and Human
Breast Cancer

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REPORT DATE: June 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Jun 00 - 31 May 01)		
4. TITLE AND SUBTITLE The Tumor Suppressor Protein TEP1/PTEN/MMAC1 and Human Breast Cancer		5. FUNDING NUMBERS DAMD17-98-1-8271		
6. AUTHOR(S) Hong Sun, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Yale University New Haven, CT 06520-8047 E-Mail: hong.sun@yale.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) We have previously cloned a novel protein, TEP1, also called PTEN or MMAC1, based on its sequence homology to members of protein tyrosine phosphatases. PTEN/MMAC1/TEP1 has now been well established as an important tumor suppressor that is commonly mutated in a wide variety of human cancers. At molecular level, PTEN/MMAC1/TEP1 protein has been shown to function as a phosphatase for phosphatidylinositol 3,4,5-trisphosphate, thus acting as a negative regulator for PI 3-kinase signaling pathway. PTEN deletions and mutations are frequently found in advanced and highly metastatic cancers, suggesting that PTEN may also play a role in preventing tumor progression and metastasis, in addition to its role as a suppressor for tumor initiation. The initiation of metastasis is usually a result of cytoskeletal rearrangements which promote enhanced cell motility. We have previously shown that genetic deletion of the Pten gene can lead to increased levels of phosphatidylinositol-3,4,5-trisphosphate and enhanced phosphorylation of Akt. We have recently discovered that these cells also contain higher levels of the activated forms of Rac1 and Cdc42. Rac and Cdc42 are two small GTPases that have been implicated in cell motility and tumor invasion processes. Our studies have provided a molecular explanation for the increased cell motility and therefore invasion associated with loss of PTEN in human breast cancer cells.				
14. SUBJECT TERMS tumor suppressors, signal transduction, metastasis, invasion, breast cancer, TEP1, PTEN, MMAC1			15. NUMBER OF PAGES 12	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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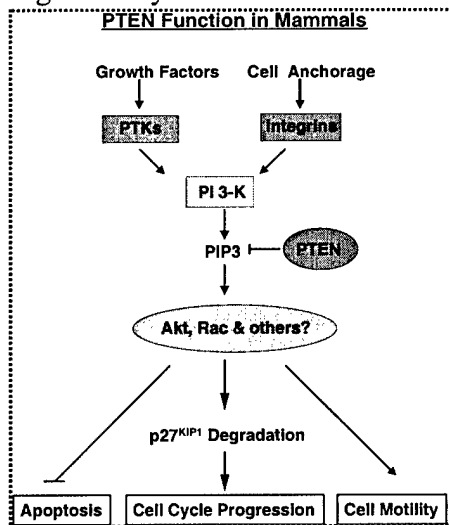
Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D-M., Zheng, Y., Sun, H. and Wu, H.
(2000) Genetic deletion of the PTEN tumor suppressor gene promotes cell motility by activation of
Rac1 and Cdc42 GTPases. *Curr. Biol.* 10, 401-404.

INTRODUCTION

We have previously cloned a novel protein, TEP1, based on its sequence homology to members in the dual specificity subfamily of protein tyrosine phosphatases [1]. The gene encoding TEP1 maps to chromosome 10q23, a locus frequently deleted in human glioblastomas and prostate cancers. The TEP1 gene was found to be identical to PTEN or MMAC1, a tumor suppressor gene independently isolated by positional cloning method [2,3]. Our group was the first to demonstrate that *in vitro* PTEN protein possesses an intrinsic phosphatase activity towards phosphotyrosine containing proteins [1].

PTEN has now been well established as an important tumor suppressor that is commonly mutated in a wide variety of human cancers. Somatic mutations in the *PTEN* gene have been found in glioblastoma, endometrial carcinoma, prostate and breast cancers, while inherited mutations have been associated with cancer predisposition syndromes such as Cowden disease and Banayan-Zonana syndrome [7]. Recent studies have suggested that the PTEN protein can dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PIP3) [8] *in vitro*. PIP3 is a product of PI 3-kinases and serves as second messengers for the activation of the serine/threonine kinase Akt, small GTPase Rac and S6 kinase [9].

In my original proposal, I have outlined strategies to investigate the involvement of PTEN in human breast cancers. In order to clearly and definitely define the contribution of PTEN deficiency to human breast cancers, we need to understand the cellular processes and the signaling pathways regulated by PTEN. We have made great progresses towards these goals. In the first funding year



for my breast cancer grant (DAMD17-98-1-8271), we have shown that demonstrated that PTEN regulates cell cycle progression at the G1/S transition in both human tumor cells and mouse *Pten* knock-out cells [7,8]. Our observation of PTEN regulating cell cycle progression provides a molecular explanation for the marked over-proliferation and increase of S-phase cell population phenotype seen in *Pten*^{-/-} mouse embryos [9]. Second, we have identified p27^{KIP1}, an inhibitor of G1 cyclin-dependent kinases, as a specific downstream target for the PTEN-regulated signaling pathway [7,8]. Emerging evidence indicates that p27 serves as a critical cell cycle regulator that mediates the growth factor-dependent cell cycle progression and entry of the S-phase. The level of p27 is also a good prognosis marker for human breast cancer. Our studies raise an interesting possibility that PTEN deficiency or haploinsufficiency may lead to down-regulation of p27 which in turn promotes breast cancer progression.

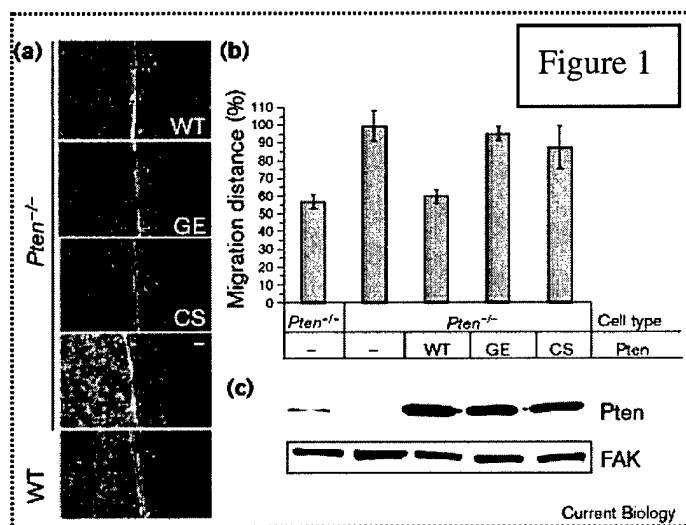
In the second funding year, our second year research has been focused on elucidation the potential role of PTEN in regulation of breast cancer cell migration and invasion. *PTEN* deletions and mutations are frequently found in advanced and highly metastatic cancers [2,3], suggesting that PTEN may also play a role in preventing tumor progression and metastasis, in addition to its role as a suppressor for tumor initiation. The initiation of metastasis is usually a result of cytoskeletal rearrangements which promote enhanced cell motility. There is growing evidence that small GTPases of the Rho family, Rac1, Cdc42 and RhoA, play a critical role in regulating the actin cytoskeleton to promote cell motility and adhesion [10]. Like all members of the Ras superfamily, Rho proteins act as molecular switches that cycle between the active GTP-bound and inactive GDP-bound states. This cycling is regulated positively by GDP/GTP exchange factors (GEFs), and negatively by GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs that activate these small GTPases all contain a pleckstrin homology (PH) domain

C-terminally adjacent to the catalytic Dbl homology (DH) domain. It has been shown that the PH domains in certain GEFs interact specifically with the membrane anchored phosphatidylinositides, which mediate their membrane localization and activation. Overexpression of constitutively activated Rac1 and Cdc42, or their corresponding exchange factors have been shown to promote cell migration. However, whether Rac1 and Cdc42 activities are modulated by the PTEN signaling pathway is yet to be established.

In collaboration with Dr. Hong Wu's laboratory in UCLA, we have employed isogenic *Pten*^{+/+} and *Pten*^{-/-} mouse fibroblasts to study the function of PTEN in regulation of tumor progression and metastasis. Our results demonstrate that Rac1 and Cdc42 are two of the downstream effectors of PTEN for cell motility, and that the lack of lipid phosphatase activity of PTEN is responsible for the increased cell migration phenotype of the *Pten*-deficient cells [11].

BODY

PTEN-deficiency promotes cell motility



We have compared *Pten*^{+/+} and *Pten*^{-/-} mouse fibroblasts (MEF) for cell motility. By using several independent assays, we found that *Pten*^{-/-} cells have increased cell motility than their wild type counterparts. In order to obtain more quantitative measurements of the migration distance, we employed a modified "wound healing" assay. In this assay, cells are first seeded on coverslips, and then the coverslips are transferred to a new plate coated with fibronectin. Upon transfer, cells migrate from the rim of the coverslip outwards onto the new plate. This method, thus, allows for accurate measurements of the migration distance over time. As shown in the Figure 1a, *Pten*^{-/-} cells migrate

almost twice as fast as *Pten*^{+/+} cells. Together, these results suggest that *PTEN* negatively regulates signaling pathways controlling cell migration.

Increased cell motility in *Pten*^{-/-} cells is due to deficiency of the PTEN lipid phosphatase activity.

PTEN protein contains multiple structural motifs besides its phosphatase domain [1-3]. The N-terminal half of the protein shares extensive homology with tensin, a cytoskeletal protein localized to focal adhesions, and auxillin, a protein involved in synaptic vesicle transport. The C-terminal of the protein contains a motif that might bind to PDZ domain-containing proteins. In order to determine whether increased cell migration in *Pten*^{-/-} cells is due to lack of the PTEN phosphatase activity or if other structural motifs may play a role, we re-introduced either wild-type (WT) or *PTEN* C124S, a catalytically inactive mutant, into the *Pten*^{-/-} cells by retrovirus - mediated gene delivery method. We used a retroviral vector that expresses the gene of interest and the green fluorescence protein (GFP) as a bicistronic mRNA. GFP was translated using an internal ribosome entry site (IRES-GFP). This expression system allowed us to sort the GFP positive, thus *PTEN* expressing cells, by fluorescence activated cell sorting (FACS) following retroviral infection. As shown in Fig. 3C, WT *PTEN* and C124S mutant were expressed in comparable levels in the sorted

populations. The sorted populations were then subjected to cell migration assays. As shown in Fig. 1a and quantified in 1b, WT *PTEN*, but not C124S mutant, could fully reverse the migration phenotype of *Pten*^{-/-} cells, confirming that the enhanced motility is directly due to the lack of PTEN phosphatase activity.

Recent studies have suggested that PIP3 is a major *in vivo* substrate for PTEN. Interestingly, *PTEN* G129E, a point mutation first being identified as an inherited mutation in Cowden disease kindreds, has been shown to be deficient for the phosphatase activity towards PIP3, while its activity towards synthetic protein substrates is unaffected. Using *PTEN* G129E mutant, we further tested whether PTEN controls cell migration through its lipid phosphatase activity or its protein phosphatase activities. As shown in Figure 1a and 1b, *PTEN* G129E behaved similar to the C124S mutant as they were both unable to rescue the migration phenotype in this assay. This experiment suggests that the enhanced motility of *Pten*^{-/-} cells is a result of the loss of PTEN phosphatase activity, in particular, its lipid phosphatase activity.

***Pten* deletion results in up-regulation of Rac1 and Cdc42 activity.**

Since increased cell motility is associated with a deficiency in PTEN lipid phosphatase activity, and cells lacking *Pten* contain elevated levels of PIP3, we next examined whether activation of known downstream PIP3 effectors might be responsible for the increased cell migration phenotype in *Pten*^{-/-} cells. Two of the most notable effectors involved in cell migration are the small GTPases Rac1 and Cdc42. Activation of Cdc42 and Rac1 has been implicated in promoting cell migration and the their GDP/GTP exchange factors (GEFs) can be activated in a PIP3-dependent manner. We

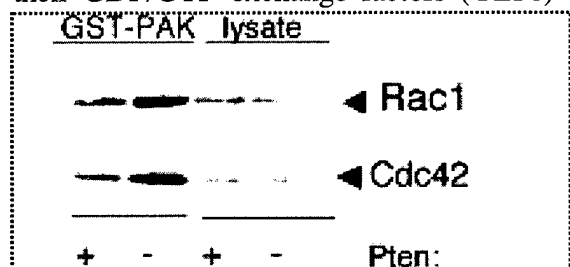


Figure 2. Increases of GTP-bound forms of Rac and Cdc42 in *Pten*^{-/-} cells.

therefore examined whether PTEN deficiency leads to a change in the Rac1 and Cdc42 activities. To determine the endogenous levels of the GTP-bound (thus the activated) forms of Rac1 or Cdc42, we adopted an affinity-based effector-Rac1 or Cdc42 GTPase co-precipitation assay. In this assay, the p21-binding domain (PBD) of PAK1, which specifically recognizes Rac1-GTP or Cdc42-GTP forms, was expressed as a GST-fusion protein to act as a bait to trap the respective GTPases in cell lysates. To confirm the specificity of this assay, we pre-loaded Rac1 and Cdc42 present in the cell

lysates with either GTPγS or GDP prior to affinity precipitation by GST or GST-PAK immobilized glutathione agarose beads. The GTP-bound forms of Rac1 and Cdc42 could form tight complexes with GST-PAK1, which could be detected by Western blot analysis with the corresponding antibodies. However, GST-PAK1 could not interact with either GTPase when they were loaded with GDP, suggesting that the affinity precipitation assay is specific and effective in assessing the activation states of Rac1 and Cdc42.

We then applied the GST-PAK precipitation assay to examine the level of endogenous GTP-bound forms of Rac1 or Cdc42 in *Pten*^{-/-} cells and *Pten*^{+/+} cells. As shown in Fig. 2 in the logarithmically growing cells, there are marked increases of the GTP-bound forms of Rac1 and Cdc42 in *Pten*^{-/-} cells compared to *Pten*^{+/+} cells, while the total protein levels of Rac1 or Cdc42 are not affected by the *PTEN* status. In order to test whether Rac1 and Cdc42 were activated in a PI3-kinase dependent manner, we also treated *Pten*^{-/-} cells with the PI3-kinase inhibitor LY294002. We have found that similar to Akt, a downstream effector of PI3-kinase, Rac1 activity dramatically decreases upon LY294002 treatment, indicating that Rac1 activation in *Pten*^{-/-} cells is dependent on enhanced PIP3 levels in these cells [11]. Together, these studies suggest that *PTEN* deficiency leads to increases of the activated forms of Rac1 and Cdc42 in *Pten*^{-/-} cells.

Dominant negative forms of Rac1 and Cdc42 reverse the migration phenotype in *Pten*^{-/-} cells.

To determine whether the elevated levels of the GTP-bound forms of Rac1 and Cdc42 in *Pten*^{-/-} cells are responsible for the increased cell migration phenotype, we introduced either WT or dominant negative forms of Rac1 (N17Rac1), Cdc42 (N17Cdc42), or RhoA (N19RhoA) into *Pten*^{-/-} cells by retroviral infection, again using the pMX-IRES-GFP vector. These mutants are thought to act by sequestering specific GEFs necessary for activation of Rac1, Cdc42, or RhoA, preventing their functions [10]. Since the infection efficiencies were more than 90% and the observed GFP expressions were at similar levels as analyzed by FACS, we used unsorted populations for the cell migration assay.

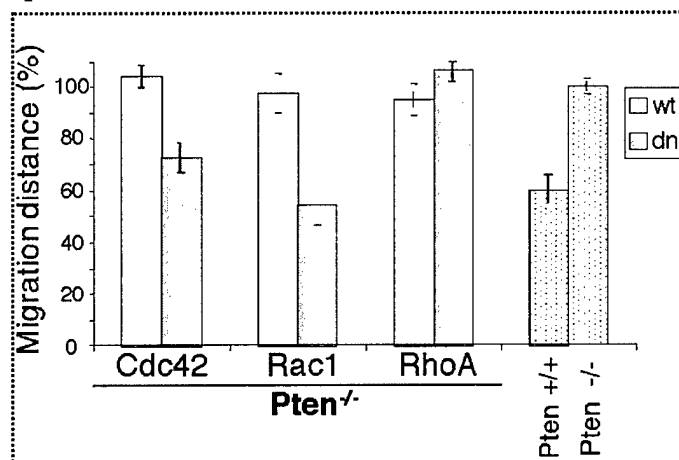


Figure 3 shows that expression of N17Rac1 and N17Cdc42 in *Pten*^{-/-} cells could reverse the cell migration phenotype by 100% and 50%, respectively. The less efficient reversion by N17Cdc42 could reflect the suggested hierarchical relationship between the Rac1 and Cdc42, where Cdc42 is thought to function upstream of Rac1. As a control for the specificity of these GTPases, we also expressed the dominant negative form of RhoA (N19RhoA). Activation of RhoA causes formation of focal adhesions and stress fibers, but since the abundance and size of these structures correlate

negatively with cell locomotion, RhoA seems more likely to be involved in adhesion than motility. No effect on the migration of *Pten*^{-/-} fibroblasts was observed with N19RhoA, nor with the wild type Rho GTPases. These results indicate that Rac1 and Cdc42 serve as downstream effectors of PTEN in the regulation of cell migration.

KEY RESEARCH ACCOMPLISHMENTS

Our studies in the past year have demonstrated that PTEN regulates cell motility through in phosphatidylinoside (3,4,5)-trisphosphate. Our findings are summarized as below:

1. Demonstrate that the in vivo PTEN regulates cell motility.
2. Demonstrate that the effect of PTEN on cell motility is mediated by its phosphatase activity on phosphatidylinoside (3,4,5)-trisphosphate.
3. Demonstrate that the downstream targets for PTEN in cell motility regulation are small GTPases Rac and Cdc42.

REPORTABLE OUTCOMES

Manuscripts

Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D-M., Zheng, Y., Sun, H. and Wu, H. (2000) Genetic deletion of the PTEN tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Curr. Biol.* 10, 401-404.

CONCLUSIONS

PTEN deletions and mutations are frequently found in advanced and highly metastatic cancers, suggesting that *PTEN* may also play a role in preventing tumor progression and metastasis, in addition to its role as a suppressor for tumor initiation. The initiation of metastasis is usually a result of cytoskeletal rearrangements which promote enhanced cell motility. We have previously shown that genetic deletion of the *Pten* gene can lead to increased levels of phosphatidylinositol-3,4,5-trisphosphate and enhanced phosphorylation of Akt. We have recently discovered that these cells also contain higher levels of the activated forms of Rac1 and Cdc42. Rac and Cdc42 are two small GTPases that have been implicated in cell motility and tumor invasion processes. Our studies have provided a molecular explanation for the increased cell motility and thus invasion associated with loss of *PTEN* in human breast cancer cells.

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Genetic deletion of the *Pten* tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases

Joanna Liliental^{*,†}, Sun Young Moon[‡], Ralf Lesche^{*§}, Ramanaiah Mamillapalli[¶], Daming Li[¶], Yi Zheng[‡], Hong Sun[¶] and Hong Wu^{*§}

***Pten* (Phosphatase and tensin homolog deleted on chromosome 10) is a recently identified tumor suppressor gene which is deleted or mutated in a variety of primary human cancers and in three cancer predisposition syndromes [1]. *Pten* regulates apoptosis and cell cycle progression through its phosphatase activity on phosphatidylinositol (PI) 3,4,5-trisphosphate (PI(3,4,5)P₃), a product of PI 3-kinase [2–5]. *Pten* has also been implicated in controlling cell migration [6], but the exact mechanism is not very clear. Using the isogenic *Pten*^{+/+} and *Pten*^{-/-} mouse fibroblast lines, here we show that *Pten* deficiency led to increased cell motility. Reintroducing the wild-type *Pten*, but not the catalytically inactive *Pten* C124S or lipid-phosphatase-deficient *Pten* G129E mutant, reduced the enhanced cell motility of *Pten*-deficient cells. Moreover, phosphorylation of the focal adhesion kinase p125^{FAK} was not changed in *Pten*^{-/-} cells. Instead, significant increases in the endogenous activities of Rac1 and Cdc42, two small GTPases involved in regulating the actin cytoskeleton [7], were observed in *Pten*^{-/-} cells. Overexpression of dominant-negative mutant forms of Rac1 and Cdc42 reversed the cell migration phenotype of *Pten*^{-/-} cells. Thus, our studies suggest that *Pten* negatively controls cell motility through its lipid phosphatase activity by down-regulating Rac1 and Cdc42.**

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Received: 23 November 1999
Revised: 4 January 2000
Accepted: 21 January 2000

Published: 24 March 2000

Current Biology 2000, 10:401–404

0960-9822/00/\$ – see front matter
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Results and discussion

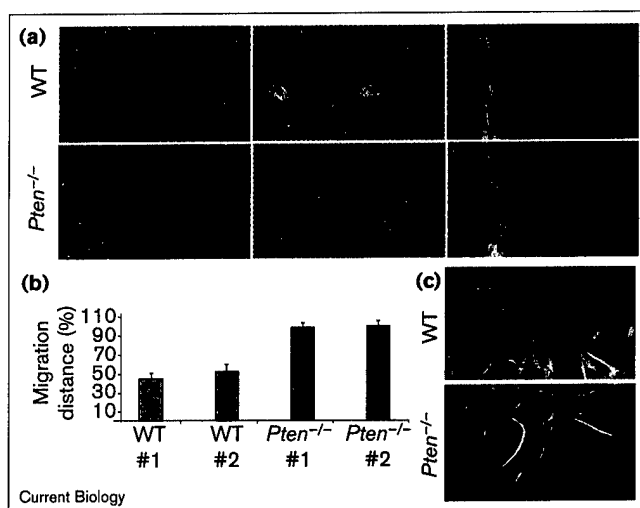
To test the role of *Pten* in cell migration, we established independent immortalized fibroblast lines from wild-type

and *Pten* deficient (*Pten*^{-/-}) mice, using the 3T9 protocol [8]. Similar to primary mouse embryonic fibroblasts [4], the immortalized *Pten*^{-/-} cell lines showed increased levels of phosphorylation of protein kinase B/Akt compared to their wild-type counterparts and were resistant to serum-deprivation induced apoptosis. In contrast to our observation with wild-type and *Pten*^{-/-} embryonic stem cells [4], however, no significant differences in the rates of cell proliferation and the levels of the cyclin-dependent kinase inhibitor p27^{KIP1} could be detected between log-phase growing wild-type and *Pten*^{-/-} fibroblast cells (data not shown).

Pten^{-/-} fibroblasts have an increased cell motility, as shown by a classic ‘wound healing’ assay (Figure 1a, left panels) [9]. They were able to completely close the wound within 15 hours, whereas wild-type cells took almost 30 hours. To demonstrate that the increase in cell migration is an individual cell based and cell division-independent event, we employed a colloidal-gold based motility assay [10]. This assay revealed that *Pten*^{-/-} fibroblasts could produce longer ‘trails’ than wild-type cells in a defined time period, indicating that *Pten*^{-/-} cells indeed migrate faster than the wild-type cells (Figure 1a, middle panels). In order to obtain more quantitative measurements of the migration distance, we employed a modified ‘wound healing’ assay. In this assay, cells are first seeded on coverslips, and then transferred to a new plate coated with fibronectin. Upon transfer, cells migrate from the rim of the coverslip outwards onto the new plate. As shown in Figure 1a (right panels) and quantified in Figure 1b with independent cell lines, *Pten*^{-/-} cells migrate almost twice as fast as wild-type cells. Moreover, careful observation of cell morphology revealed that *Pten*^{-/-} fibroblasts appeared rounded and had intense cortical F-actin staining (Figure 1c). Together, these results suggest that *Pten* negatively regulates signaling pathways controlling cell migration.

In order to determine whether increased cell migration in *Pten*^{-/-} cells is due to lack of the *Pten* phosphatase activity or if other structural motifs may play a role, we re-introduced either wild-type *Pten* or *Pten* C124S, a catalytically inactive mutant, into the *Pten*^{-/-} cells by retrovirus infection [11]. We used a retroviral vector that expresses the gene of interest and the green fluorescent protein (GFP) as a bicistronic mRNA. GFP-positive, thus *Pten*-expressing, cells were sorted by fluorescence activated cell sorting (FACS) following retroviral infection. Wild-type *Pten* and the C124S mutant were expressed in comparable levels in the sorted populations (Figure 2c). As shown in Figure 2a

Figure 1

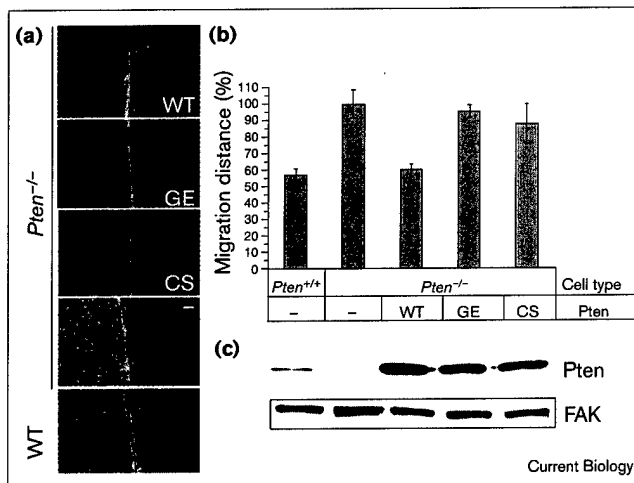


Pten-deficient fibroblasts migrate faster than wild type. (a) An equal number of wild-type (WT) or *Pten*^{-/-} fibroblasts were seeded on a fibronectin-coated plate and cultured for 24 h. Migration into the wound is shown 15 h after the wound was introduced (left panels; open arrowheads point to the boundaries of the wound at time = 0). In the middle panel, 2×10^3 cells per well were seeded on colloidal gold-coated 6-well dishes in duplicates. Migration of wild-type or *Pten*^{-/-} fibroblasts is shown at 24 h. The scale bar represents 10 μ m. In the right panels, wild-type or *Pten*^{-/-} fibroblasts grown on glass coverslips were placed onto 5 μ g/ml fibronectin-coated dishes and cultured for 15 h. (b) Cell motility was assessed and compared using independent cell lines. Migration distances were determined by taking seven independent measurements from each coverslip. Each experiment was conducted in triplicate, and mean \pm SD was calculated. The migration distance is normalized so that 100% represents migration distance of *Pten*^{-/-} cells. (c) *Pten*^{-/-} cells exhibited increased cortical actin polymerization as compared to the wild-type cells. Briefly, log-phase growing fibroblasts were cultured without serum for 20 h. After fixation in 4% paraformaldehyde, cells were permeabilized with 0.2% Triton X-100 and stained for F-actin using rhodamine-phalloidin (Molecular Probes).

and quantified in Figure 2b, wild-type *Pten*, but not the C124S mutant, could fully reverse the migration phenotype of *Pten*^{-/-} cells, confirming that the enhanced motility is directly due to the lack of Pten phosphatase activity.

Recent studies suggested that PI(3,4,5)P₃ is a major substrate for Pten both *in vitro* [2] and *in vivo* [3,4]. Interestingly, *Pten* G129E is deficient for the phosphatase activity towards PI(3,4,5)P₃, while its activity towards synthetic protein substrates is unaffected [12]. Using the *Pten* G129E mutant, we further tested whether Pten controls cell migration through its lipid phosphatase activity or its protein phosphatase activities. *Pten* G129E behaved similarly to the C124S mutant as they were both unable to rescue the migration phenotype in this assay. This experiment suggests that the enhanced motility of *Pten*^{-/-} cells is a result of the loss of Pten phosphatase activity, in particular, its lipid phosphatase activity.

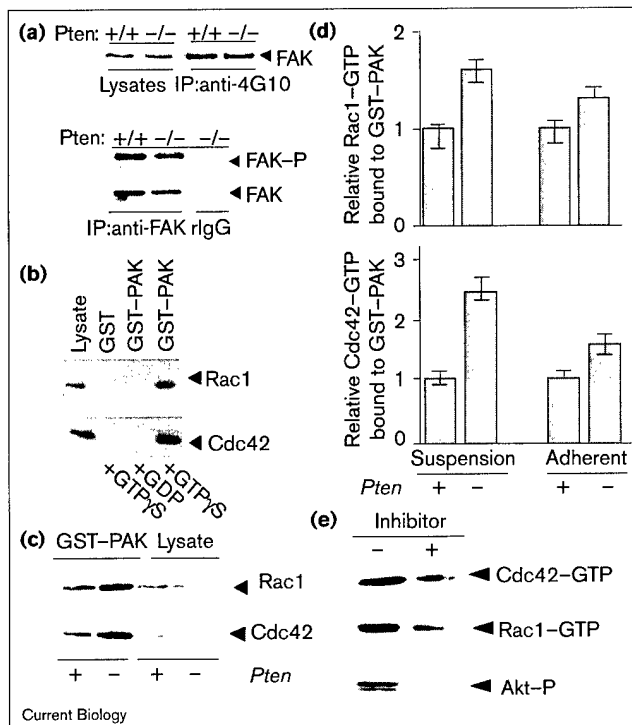
Figure 2



Increased cell motility in *Pten*^{-/-} cells is due to the lack of lipid phosphatase activity of *Pten*. (a) *Pten*^{-/-} cells were infected with retroviral GFP vectors containing wild-type *Pten* (WT), G129E (GE), or C124S (CS) *Pten* mutants. Control wild-type or *Pten*^{-/-} cells were infected with viruses containing GFP only. 48 h later, GFP positive cells were sorted by fluorescence-activated cell sorting (FACS), seeded onto glass coverslips in triplicate, and grown for an additional 5 h. Cells on coverslips were then replaced onto a fibronectin-coated surface and incubated for 15 h. (b) Quantitative representation of (a). Cell migration is normalized so that 100% represents the migration distance of *Pten*^{-/-} cells infected by empty vector. (c) Pten protein levels in uninfected and infected cells after FACS sorting. Western blots of total protein extracts were probed with an affinity-purified anti-Pten antibody. Blots were reprobed with anti-FAK antibody (Santa Cruz Biotechnology) to confirm equal loading.

It has been suggested that Pten negatively regulates cell migration by directly dephosphorylating p125^{FAK} and changing mitogen-activated protein (MAP) kinase activity [6,13]. In order to test whether p125^{FAK} phosphorylation and MAP kinase activation are also affected by the loss of *Pten*, we examined the tyrosine phosphorylation status of these proteins. Whole cell lysates from log-phase growing wild-type or *Pten*^{-/-} fibroblasts were immunoprecipitated with 4G10 anti-phosphotyrosine antibody and western blotted with anti-FAK antibody (Figure 3a, upper panel), or were immunoprecipitated with anti-FAK antibody and western blotted with 4G10 (Figure 3b, lower panel). In contrast to what would be predicted if Pten could directly dephosphorylate p125^{FAK}, no difference in tyrosine phosphorylation of p125^{FAK} could be detected in *Pten*^{-/-} fibroblast lines compared to wild-type cells. The activation status of MAP kinases was not affected by the *Pten* deletion either, but the level of Akt phosphorylation was significantly increased in *Pten*^{-/-} fibroblast cell lines (data not shown), similar to what we have observed previously in *Pten*^{-/-} embryonic stem cells [4]. These results suggest that the enhanced cell motility caused by *Pten* deficiency may be mediated by effectors other than p125^{FAK} and MAP kinases.

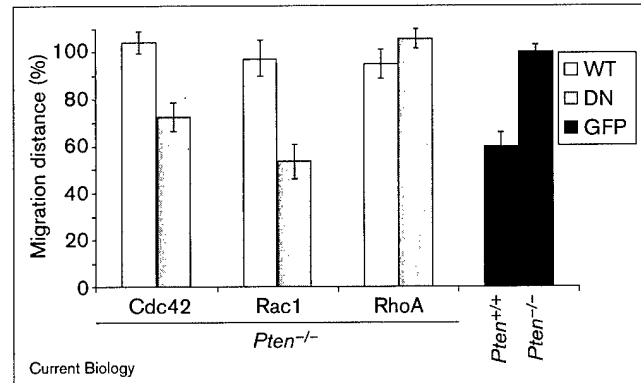
Figure 3



Pten deletion results in activation of Rac1 and Cdc42, but not FAK. (a) FAK phosphorylation is not affected by *Pten* deletion. Upper panel, equal amounts of proteins were immunoprecipitated with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology). Western blots were probed with anti-FAK antibody. Bottom panel, anti-FAK immunoprecipitates were western blotted with 4G10 (for phosphorylated FAK, FAK-P) or an anti-FAK antibody. rlgG indicates immunoprecipitation with a rabbit isotype control antibody. (b) GST-PAK binding is specific for GTP. *Pten*^{-/-} fibroblast lysates were loaded with GTPγS or GDP prior to affinity precipitation with GST or GST-PAK immobilized on 10 μg glutathione-agarose beads. The precipitated proteins were analyzed by western blot with anti-Rac1 (Upstate Biotechnology) or anti-Cdc42 (Santa Cruz Biotechnology) antibody, respectively. Results are representative of three independent experiments. (c) The GST-PAK precipitates from wild-type (+) and *Pten*^{-/-} (-) fibroblasts under log phase growing conditions, along with total cell lysates, were analyzed by western blot with anti-Rac1 or anti-Cdc42 antibodies. (d) Wild-type or *Pten*^{-/-} cells, either in suspension or adherent, were lysed and subjected to GST-PAK affinity precipitation analysis. The Rac1-GTP (upper graph) or Cdc42-GTP (lower graph) activities detected by western blot were normalized to the amount of Rac1 or Cdc42 in whole cell lysates. Results are means ± SD from three experiments. (e) *Pten*^{-/-} cells were treated with 20 μM LY294002 (shown here, +), or 50 nM wortmannin (not shown), or DMSO vehicle control (-) for 6 h before harvesting. Cell lysates were precipitated with GST-PAK and blotted with anti-Rac1 or anti-Cdc42 antibodies, respectively. In parallel, aliquots of cell lysates were analyzed with anti-phospho-Akt antibody (New England Biolabs).

As increased cell motility is associated with a deficiency in *Pten* lipid-phosphatase activity, and cells in which *Pten* is genetically deleted contain elevated levels of PI(3,4,5)P₃ [3,4], we next examined whether activation of known

Figure 4



Increased motility of *Pten*^{-/-} cells can be reversed by expression of dominant-negative (DN) Rac1 and Cdc42, but not RhoA. Cell motility was assessed by directly measuring the migration distance 15 h after plating, and presented as an average of three independent experiments. WT, wild-type constructs were expressed; GFP, a GFP-expressing vector only was expressed.

downstream PI(3,4,5)P₃ effectors might be responsible for the increased cell migration phenotype in *Pten*^{-/-} cells. Activation of Cdc42 and Rac1 has been implicated in promoting cell migration [7] and their GDP/GTP exchange factors (GEFs) can be activated in a PI(3,4,5)P₃-dependent manner [14,15]. We therefore examined whether *Pten* deficiency leads to changes in the Rac1 and Cdc42 activities. In this assay, the p21-binding domain of PAK1 was expressed as a GST-fusion protein. GST-PAK1 can specifically recognize Rac1-GTP or Cdc42-GTP forms, but not GDP-bound forms, suggesting that the affinity precipitation assay is specific and effective in assessing the activation states of Rac1 and Cdc42 (Figure 3b). We then examined the level of endogenous GTP-bound forms of Rac1 or Cdc42 in *Pten*^{-/-} cells and wild-type cells. As shown in Figure 3c, there are marked increases of the GTP-bound forms of Rac1 and Cdc42 in logarithmically growing *Pten*^{-/-} cells compared to wild-type cells, although the total protein levels are not affected by the *Pten* status. As PI(3,4,5)P₃ levels were highly sensitive to growth conditions [4], we also examined the Rac1 and Cdc42 activities in unfavorable confluent culture conditions. There is a notable ~30% increase in Rac1-GTP content and a ~50% increase in Cdc42-GTP content compared to the wild-type cells (Figure 3d). When similar assays were performed using suspended cell cultures which lack the adherent stimuli, ~60% and ~130% increases of Rac1-GTP and Cdc42-GTP forms, respectively, were observed (Figure 3d, suspension). The extent of elevation in the endogenous Cdc42 and Rac1 activities in *Pten*^{-/-} cells were consistent when independent cell lines were used, and reintroducing wild-type *Pten* into *Pten*^{-/-} cells led to a decrease in the GTP-bound forms of Rac1 and Cdc42 (data not shown).

Recent experiments demonstrated that a correlation exists between activation of the activity of PI 3-kinase and the activities of Rac1 and Cdc42 [14–17]. However, whether PI 3-kinase functions downstream or upstream of Rac1 and Cdc42 remains unclear. To test whether Rac1 and Cdc42 were activated in a PI 3-kinase dependent manner, we treated *Pten*^{-/-} cells with the PI 3-kinase inhibitor LY294002 or wortmannin. Figure 3e shows that the activities of Rac1 and Cdc42 dramatically decrease upon treatment with PI 3-kinase inhibitors, indicating that similar to Akt, Rac1 and Cdc42 activation in *Pten*^{-/-} cells is downstream of PI 3-kinase.

To formally prove that the elevated endogenous activities of Rac1 and Cdc42 in *Pten*^{-/-} cells are responsible for the increased cell migration phenotype, we introduced either wild-type or dominant negative forms of Rac1 (N17Rac1) and Cdc42 (N17Cdc42) into *Pten*^{-/-} cells by retroviral infection. These mutants are thought to act by sequestering specific GEFs necessary for activation of Rac1 and Cdc42, preventing their functions. Figure 4 shows that expression of N17Rac1 and N17Cdc42 in *Pten*^{-/-} cells could reverse the cell migration phenotype by 100% and 50%, respectively. The less efficient reversion by N17Cdc42 is not due to the lower expression level (data not shown), but could reflect the suggested hierarchical relationship between Rac1 and Cdc42, where Cdc42 is thought to function upstream of Rac1 [7]. As a control for the specificity of these GTPases, we also expressed the dominant negative form of RhoA (N19RhoA), a GTPase involved in focal adhesion and stress fiber formation [18,19]. No effect on the migration of *Pten*^{-/-} fibroblasts was observed with N19RhoA, or with the wild-type Rho GTPases (Figure 4). These results indicate that Rac1 and Cdc42 serve as downstream effectors of Pten in the regulation of cell migration.

In summary, we show that inactivation of the *Pten* tumor suppressor gene promotes cell motility in fibroblasts. In contrast to previous reports that Pten negatively regulates cell migration by directly dephosphorylating p125^{FAK} and changing MAP kinase activities, we demonstrate genetically that the tumor suppressor Pten controls cell motility by down regulating Rac1 and Cdc42 GTPases, and this negative regulation is dependent on the lipid phosphatase activity of Pten. In combination with our previous work and other studies, we suggest that Pten exerts its tumor suppressor function not only at the stage of tumor initiation, but also in tumor progression and metastasis.

Supplementary material

Supplementary material including additional methodological details is available at <http://current-biology.com/supmat/supmatin.htm>.

Acknowledgements

We thank H. Herschman, O. Witte, C. Sawyers, K. Shuai, X. Liu for critical reading of the manuscript. We thank X.L. Liu and H.L. Lodish of MIT for kindly providing pMX-IRES-GFP vector, and Jing Gao and Nadia Gavrilova

for technical assistance. R.L. is supported by the Deutsche Forschungsgemeinschaft and a Carolan Seed grant (to H.W.). H.S. is a Pew Scholar in the Biomedical Sciences. H.W. is an Assistant Investigator of the Howard Hughes Medical Institute and V Foundation Scholar. This work was supported by the V foundation and a Carolan Seed grant (to H.W.); American Cancer Society (RPG-97-146) and National Institutes of Health grant (GM53943 to Y.Z.); Department of the Army (DAMD 17-98-1-8271) and National Institutes of Health grant (CA77695 to H.S.).

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